AD_____

Award Number: DAMD17-00-1-0006

TITLE: The Role of Maspin in Prostate Tumorigenesis and Development

PRINCIPAL INVESTIGATOR: Ming Zhang, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

REPORT DATE: February 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2003	Annual (15 Jan 02 - 14 Jan 03)

4. TITLE AND SUBTITLE
The Role of Maspin in Prostate Tumorigenesis and Development

5. FUNDING NUMBERS
DAMD17-00-1-0006

6. AUTHOR(S) :
Ming Zhang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-Mail:mzhang@bcm.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The object of this proposal is to understand the tumor suppressor function of maspin in prostate, and to explore maspin's role in normal prostate development. Maspin knockout mouse model will be employed to study the effects of loss of maspin function on mouse prostate tumorigenesis and development. We hypothesize that the presence of maspin (by stable transfection or by adenovirus mediated maspin gene delivery) will prevent or delay prostatic tumorigenesis and metastasis, while loss of maspin in mouse model will render it more susceptible to tumor formation and metastasis. We will take advantage of the powerful tool of mouse genetics by crossing maspin knockout mice with a well characterized mouse prostate cancer model (TRAMP) to test this hypothesis. Prostatic tumorigenesis and normal prostate development will be studied using a variety of established techniques, including organ culture, histopathology, and molecular biology.

14. SUBJECT TERMS:
prostate cancer, maspin, tumorigenesis, metastasis

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

___ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

___ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

___ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

___ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Ming Zhang, Ph.D. 1/23/03

PI - Signature Date

Table of Contents

Introduction

Prostate cancer is the most commonly diagnosed cancer in U.S. males. Prostate cancer incidence rates increased 141.8% between 1973 and 1994. A life is lost to prostate cancer in this country every 13 minutes. In 1998, about 39,200 men died of prostate cancer. Due to the severity of this malignant disease, research of prostate needs to be focused on the treatment of prostate cancer and scientists in this field are obligated to accelerate the process of translation of their basic research into clinical usage.

The object of this proposal is to understand the tumor suppressor function of maspin in prostate, and to explore maspin's role in normal prostate development. Maspin knockout mouse model will be employed to study the effects of loss of maspin function on mouse prostate tumorigenesis and development. We hypothesize that the presence of maspin (by stable transfection or by adenovirus mediated maspin gene delivery) will prevent or delay prostatic tumorigenesis and metastasis, while loss of maspin in mouse model will render it more susceptible to tumor formation and metastasis. We will take advantage of the powerful tool of mouse genetics by crossing maspin knockout mice with a well-characterized mouse prostate cancer model (TRAMP) to test this hypothesis. Prostatic tumorigenesis and normal prostate development will be studied using a variety of established techniques, including organ culture, histopathology, and molecular biology.

The specific aims for this three-year proposal are:

Aim 1. Examination of maspin as a tumor suppressor in prostate. I plan to test whether maspin inhibits tumor growth in cell culture and tumor progression in athymic mice. We will deliver maspin to prostate tumors by adenovirus mediated gene delivery technique. In this way, the effectiveness of maspin as a therapeutic agent can be directly evaluated.

Aim 2. Examination of maspin knockout mice on tumor progression and normal prostate development. The effect of maspin gene disruption on prostatic tumorigenesis will be tested by crossing maspin KO mice with TRAMP mice. Maspin knockout mice will also be used to evaluate the loss of maspin on prostate development. The KO mice will be valuable to support our hypothesis if these mice are more susceptible to tumorigenesis and metastasis because of the loss of tumor suppressor, maspin.

Body

Materials and methods

Animals

Syngeneic C57BL/6 mice (for implantation of TRAMP tumor cells) were purchased from Harlan, Inc. All animals were maintained within the PI's animal facility at Baylor.
TRAMP cell lines were obtained from Dr. Norm Greenberg at Baylor College of Medicine.

Antibodies

Polyclonal anti-maspin antibody was made by Zymed, Inc. as a custom service. All secondary antibodies were purchased from Zymed, Inc.

Northern and Western analysis

RNAs and proteins were isolated from cells, prostate tissues. Total RNAs were isolated using Gibco/BRL Trizol regent. For northern blot, roughly 20 ug RNA will be loaded each lane. For Western blot analysis, protein extracts were prepared by lysing the cells in RIPA buffer. Total 100 ug protein extract will be loaded for electrophoresis.

Immunohistochemical analysis

Prostate tissues were removed from male mice and dissected. Tissues were fixed in 10% neutral formalin buffer and embedded in paraffin and sectioned at 5 μm. For maspin immunostaining, tissues were boiled in citrate buffer (Zymed, Inc.) for ten minutes for antigen retrival. The antibody was produced in rabbit against a fifteen amino acid peptide located in the reactive site loop of maspin (AbS4A). The antibody was purified using an AbS4A sulfo-linked affinity column (Sulfolink kit, Pierce, IL). The sections were stained with the affinity purified maspin antibody at a dilution of 1:400, followed by a secondary goat anti-rabbit antibody staining, and the color was developed by Zymed's AEC chromogen kit. For specific peptide blocking, a concentration of 10 nM of AbS4A peptide was preincubated with antibody for thirty minutes at room temperature. For PCNA staining, a PCNA staining kit was purchased from Zymed (Zymed, Inc., CA) and slides were stained following the instruction of the kit.

Results and Discussion

Task 1. Examination of maspin as a tumor suppressor in prostate.

We have overexpressed maspin in TRAMP prostate tumor cells by retrovirus infection. Retroviral stable transfectants were constructed. The plasmid constructs pS2-maspin GFP and pS2-GFP were transfected into 293T cells, along with pECO plasmid using fugene reagent, to produce infective viral particles. The viral supernatants were then allowed to infect C2N TRAMP tumor cells in the presence of polybrene. The transduced cells were then selected in presence of 100 ug/ml of zeocin for four weeks. These cells were subsequently sorted by flow cytometry for green fluoroscence emitted by GFP. The retroviral stable transfectants were further selected as individual clones from 96 well titer plates. These retroviral stable transfectants were then analyzed for the presence of human maspin cDNA by RT-PCR. The expression of maspin cDNA was detected by Western blotting by mouse monoclonal antibody to human maspin (Pharmigen) and by immuno staining with ABS4a antibody to mouse maspin. These retroviral stable transfectants will be further characterized by *in vitro* experiments, by comparing the maspin transfectants versus the vector-alone transfectants and parental C2N cells, for their growth rate, anchorage-independent growth on soft agar, and tumorigenicity. The tumor cell invasion and metastasis will be compared *in vitro* by cell adhesion and motility assays. We will also analyze the changes in cell adhesion in the presence of various extracellular matrix

(ECM) proteins and thereby investigate their ability to attach to the basement membrane. This study has been published in the Journal of Urology (in press).

We have carried out in vivo experiment to examine the tumorigenic potential of these transfectants in syngenic mice. For this purpose, parental C2N cells and maspin stable cells are injected subcutaneously into C57BL/6 mice. Tumor growth rate in vivo will be monitored and samples are taken for analysis. Surprisingly, most of C2N cells inoculated developed into palpable tumors. However, none of the maspin stable clones have developed any tumors after extended time of observation, demonstrating those maspin functions to inhibit prostate tumor in vivo. We are currently identifying the mechanism of tumor suppression in prostate cancer.

Task 2. The effect of maspin gene disruption on prostate development

Because homozygous deletion of maspin is lethal, we decided to assess the partial loss of maspin on tumor progression. We hypothesize that maspin heterozygous mice may display gene dosage phenotype in prostate. This hypothesis is supported by the fact that maspin heterozygote female has a phenotype in the ovary (Zhang et al, unpublished data).

Maspin heterozygotes were crossed. Newborn male mice at day 2-day 8 were dissected for anterior and ventral prostate. The number of branching was counted. Preliminary data showed that maspin KO mice had slowed rate of prostate development. We are currently examined whether this is due to hormonal defect such as a defect in the production of androgen.

We are also going to examine the prostate in old maspin KO mice. The goal is to find out whether loss of maspin can lead increased tumorigenesis. Samples were harvested at the moment for such analysis.

Key research accomplishments

Key personnel have been recruited to initiate the study as proposed in the grant. We have established the role of maspin in suppressing prostate tumor development. One paper has been published on this study. The in vivo experiment has also been done and a manuscript is being prepared. We are currently focusing to study the effect of maspin deletion on prostate development.

Reportable outcome

1. Maspin functions as a tumor suppressor by increasing cell adhesion to extracellular matrix in prostate tumor cells. Shaji Abraham, Weiguo Zhang, Norm Greeenberg, and Ming Zhang. J. of Urology, 2003, in press.
2. Maspin inhibits prostate tumor growth in vivo. Heidi Shi, and Ming Zhang. In preparation.

Conclusion

Two tasks proposed in the grant were initiated in the first year of proposal. Key personnel have been recruited to work on the prostate project. We have established maspin stable expressing clones in TRAMP prostate tumor cells and set up animals for genetic crossing. Continuation of the tasks in the next few years will help us understand the role of maspin in

tumor metastasis and angiogenesis, and hopefully leading to the development of new therapies for the treatment of prostate cancer.

Reference

None

Appendices

Manuscript for J.U.

0022-5347/03/1693-0001/0
THE JOURNAL OF UROLOGY®

Vol. 169, ●●●-●●●, March 2003
Printed in U.S.A.
DOI: 10.1097/01.ju.0000040245.70349.37

MASPIN FUNCTIONS AS A TUMOR SUPPRESSOR BY INCREASING CELL ADHESION TO EXTRACELLULAR MATRIX IN PROSTATE TUMOR CELLS

SHAJI ABRAHAM, WEIGUO ZHANG, NORMAN GREENBERG* AND MING ZHANG

From the Baylor College of Medicine, Department of Molecular and Cellular Biology, Houston, Texas

ABSTRACT

Purpose: Maspin, a unique member of the serine protease inhibitor family, shows tumor suppressing activity for breast cancer progression and metastasis. Few studies have directly linked maspin function to prostate cancer. We used prostate tumor cells derived from the TRAMP (transgenic adenocarcinoma of mouse prostate) prostate tumor model to study the tumor suppressive function of maspin in prostate cancer.

Materials and Methods: Maspin cDNA was introduced via a retroviral plasmid into TRAMP C2N prostate tumor cells, which are aggressive and invasive in nature. We investigated the tumorigenesis of these stable cell lines in vitro by assessing the growth rate, anchorage independence and adhesion to extracellular matrix proteins such as fibronectin and laminin.

Results: Stable cell lines expressing maspin had decreased tumorigenic potential, as assessed by anchorage independent growth in soft agar assay compared with controls. Maspin stable transfectants showed decreased metastatic potential, as evaluated by modified Boyden chamber assay and increased adhesion to fibronectin and laminin.

Conclusions: Our study confirms that maspin has a tumor suppressive role not only in breast cancer, but also in prostate cancer. The data in this study suggest that maspin can decrease the tumorigenic and metastatic potential of prostate tumors, most probably by remodeling cell-extracellular matrix interactions or triggering extracellular matrix mediated signaling pathways that negatively regulate tumor migration and invasion.

KEY WORDS: prostate, prostatic neoplasms, neoplasm metastasis, cell adhesion, disease progression

Prostate cancer is one of the leading cancer related deaths in men older than 55 years.[1] Essentially a thorough understanding of the disease progression at the molecular level is important for alleviating and achieving an effective cure. In vitro prostatic cells and in vivo animal models serve as good model systems to identify prognostic markers for early disease detection and find therapeutic agents for treatment.[2] We investigated the effect of maspin effect in prostate tumors in vitro in the TRAMP (transgenic adenocarcinoma of mouse prostate) animal model.[3]

Maspin, initially identified from normal mammary epithelial cells, is a unique member of the serine protease inhibitor family that has been found to inhibit breast tumor development.[4] Among the serine protease inhibitor proteins maspin is considered a class II tumor suppressor gene,[5] since the gene is not mutated or deleted, but transcriptionally down-regulated in breast cancer.[6] Several studies have alluded to the tumor suppressive effects of maspin. For example, recombinant maspin protein inhibits breast tumor cell migration and invasion.[7,8] Zhang et al observed the inhibitory effect of maspin on angiogenesis in rat cornea and in a xenograft model.[9] High maspin expression was associated with the absence of lymph node metastasis and better overall survival in oral squamous cell carcinoma.[10] Finally the inhibition of human breast cancer by peroxisome proliferator activated receptor-γ, a differentiation agent, and γ linolenic acid was associated with up-regulation of the maspin gene.[11,12] These studies suggest the significance of maspin for negating tumor development.

Several animal studies done at our laboratory implicate the direct involvement of maspin in the suppression of primary tumor and metastasis.[4,13] When transgenic mice over expressing maspin in mammary epithelial cells under the control of mammary specific whey acidic protein promoter[14] were crossed with a strain of oncogenic whey acidic protein-simian virus 40 T antigen mice, the resulting bitransgenic mice showed inhibited tumor growth and metastasis.[13] In addition, when we tested the tumor suppressing activity of maspin in a TM40D syngeneic model in which TM40D cells or maspin transfectants were implanted back into a mammary gland, we found that the tumor growth and metastasis rates were significantly decreased in maspin transfectants.[15] These data demonstrate that maspin blocks primary tumor growth and metastasis in immunocompetent animals.[15,13]

Despite much data supporting the tumor suppressing role of maspin in breast cancer few studies have directly linked maspin function in prostate cancer. For instance, maspin gene expression is down-regulated in prostate cancer cells[16] as well as in clinical prostate cancer specimens.[17] Recombinant maspin made in vitro inhibits prostatic cell migration.[8] However, Umekita et al noted that rat maspin did not inhibit prostate tumor progression in rat derived cells.[18] Since in the TRAMP prostate animal model tumors develop that closely resemble human prostatic disease,[3] we examined the role of maspin in mouse prostate tumor cells. Thus, we introduced the maspin gene into C2N TRAMP prostate tumor cells, a highly tumorigenic and invasive cell line,[19] by a retrovirus approach.

In this study we investigated the consequence of maspin expression in TRAMP cell lines as a prelude to elucidating its function in vivo in prostate tumors. In particular we were interested in identifying whether maspin could decrease the tumorigenic and metastatic potential of C2N tumor cells. We

Accepted for publication September 13, 2002.
Supported by DAMD17–0010006 and CA79736.
* Financial interest and/or other relationship with Astra Zeneca, Genzyme, Amgen and Encore Pharmaceuticals.

to M.Z.

report that maspin can inhibit tumor invasion and increase cell-extracellular matrix (ECM) adhesion of invasive C2N tumor cells. Thus, maspin functions as a tumor suppressor, probably by remodeling cell-ECM interaction, in this prostate cancer model.

MATERIALS AND METHODS

Cell lines and tissue culture conditions. C2N cells were grown in Dulbecco's modified Eagle's medium (DMEM) without Na pyruvate supplemented with 50% heat inactivated fetal bovine serum and 50% Nu serum (Becton Dickinson, Sunnyvale, California), 0.0001 M. dihydrotestosterone, 0.05% insulin (Sigma Chemical Co., St. Louis, Missouri), and 0.05% penicillin and streptomycin at 37C in 5% CO_2. Human embryonic 293T cells were grown in DMEM containing 10% heat inactivated fetal bovine serum at 37C in 5% CO_2.

Plasmid constructs. The retroviral plasmid pS2-green fluorescent protein (GFP) was constructed by inserting GFP-zeocin from pTracer-CMV (Invitrogen, Carlsbad, California) into pS2, a retroviral plasmid containing the 5′ and 3′ long terminal repeat regions.[20] The 1.2 kb human maspin cDNA was amplified by polymerase chain reaction (PCR), filled in with Klenow enzyme and cloned into pS2-GFP vector at the Apa I site. The resultant plasmid construct was named pS2-GFP maspin.

Establishment and selection of stable transfectants. The retroviral plasmid constructs pS2-GFP or pS2-GFP maspin (10 μg.) were transfected into 293 T cells with 10 μg. pCL-Eco plasmid using fugene reagent, as described by Navaiaux et al.[21] After 24 hours C2N TRAMP cells were grown in the presence of polybrene (10 mg./ml) and viral particles for 3 hours. Virus containing medium was then replaced by fresh medium. The cells were allowed to grow for a week and then GFP or GFP-maspin stable transfectants were selected in growth medium containing 100 μg./ml. zeocin for 2 weeks. Stable transfectants were further sorted by an Altra (Beckman-Coulter, Inc., Chaska, Minnesota) flow cytometry and pools of GFP expressing cells were obtained. Control GFP expressing cells were confirmed for green fluorescence by microscopy using a model DMRB (Leica Mikroskope und Systeme GmbH, Wetzlar, Germany). GFP-maspin expressing cells were plated into 96-well plates and subclones of stable transfectants were isolated. Reverse transcriptase (RT)-PCR, Western blotting and immunostaining experiments confirmed the presence of the GFP-maspin gene in the stable transfectants.

Western blot analysis. The cells were lysed in RIPA buffer containing protease inhibitors (aprotinin, leupeptin and phenylmethylsulfonyl fluoride) and 50 μg. protein were size fractionated on 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gel. The proteins were transferred onto a polyvinylidine difluoride membrane (BioRad Laboratories, Hercules, California), blocked in 5% nonfat dry milk overnight and immunoblotted with maspin antibodies (Pharmingen, San Diego, California). With the signal amplified by secondary antibody linked to horseradish peroxidase it was detected by an enhanced chemiluminescence kit (Pierce, Rockford, Illinois). The blots were stripped and re-probed with β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, California) to confirm equal sample loading.

Cell proliferation assay. Stable GFP or GFP-maspin C2N transfectants were plated at a density of 1×10^3 cells per well in a 24-well plate in triplicate. At indicated time points of 1, 2, 4, 6 and 8 days growth was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega, Madison, Wisconsin) assay according to the manufacturer protocol. Absorbance was recorded at 420 nm. using an MRX (Dynex Technologies, Inc., Chantilly, Virginia) model microplate reader.

Cell adhesion assay. Stable GFP and GFP-maspin transfectants were harvested, washed with 1 × phosphate buffered saline, re-suspended in serum free medium with bovine serum albumin (10 mg./ml.) and plated at a density of 1×10^3 cells per well in a 24-well plate pre-coated with laminin (25 μg./ml.) or fibronectin (25 μg./ml.). The cells were incubated at 37C and 5% CO_2 for 2 hours. Nonadherent cells were removed by aspiration and adherent cells were washed 3 times with 1 × phosphate buffered saline. Total cell associated protein was determined by adding 200 μl. BCA (Pierce) working solution directly to the wells, incubating at 60C for 2 hours and lysing with 0.1% sodium dodecyl sulfate. The absorbance of each well was determined at 562 nm. in an MRX model microplate reader. Experiments were repeated 3 times in triplicate.

Colony formation in soft agar. For this assay the bottom layer contained 0.6% agarose in DMEM, while the top layer contained 0.3% agarose. Stable transfectants were seeded at a density of 6,000 cells per well in a 6-well plate in triplicate. The plates were incubated at 37C with 5% CO_2 for 3 weeks and then stained with p-iodonitrotetrazolium violet (1 mg./ml.) for 16 hours at 37C. Colonies greater than 1 mm. were counted under an inverted microscope (Leica). Colony formation was assessed in 3 independent experiments.

Cell invasion assay. Cell invasion was performed in a modified Boyden chamber assay. Briefly, the inner chamber of Millicell (Millipore, Bedford, Massachusetts) polycarbonate filters (pore size 8 μm.) coated with 2.5 mM. Matrigel in serum-free DMEM was dried overnight at room temperature under ultraviolet light. The pre-coated filters were placed in 24-well plate that formed the lower chamber and the lower chamber was filled with DMEM containing 10% serum. Cells were seeded at a density of 6×10^4 cells per well and incubated at 37C for 4 hours with 5% CO_2. After incubation cells remaining in the inner chamber were removed with a cotton swab. Cells on the outer surface of the inner chamber were fixed with 3% glutaraldehyde solution and stained with hematoxylin and eosin. Cells that penetrated the inner chamber were counted using an IX70 (Olympus Optical Corp., Melville, New York) phase contrast microscope. Experiments were performed in triplicate and repeated twice.

Statistical analysis. All statistical significance was determined by Student's t test with $p < 0.05$ considered statistically significant. Graphic presentation of the data was done using Excel (Microsoft, Seattle, Washington) spread sheet software.

RESULTS

Stable expression of maspin in C2N TRAMP cells. C2N prostate tumor cells are highly invasive and tumorigenically isolated from a primary prostate tumor in TRAMP mice. In this cell line maspin expression was not detected by RT-PCR and Western blot analysis (data not shown). To re-express maspin we transfected retroviral vectors pS2-GFP or pS2-GFP maspin into C2N cells. Zeocin resistant cells were sorted by flow cytometry and pools of GFP expressing cells were isolated (data not shown). Stable transfectants expressing variable levels of maspin were subcloned. A total of 52 single cell clones expressing variable levels of maspin were identified and representative clones were further characterized. They were examined for maspin mRNA expression by RT-PCR and for protein by immunostaining (data not shown). The level of maspin expression was quantitated by Western blot analysis. Figure 1 shows that 1 representative clone (clone 2) expressed a low level of maspin and 2 (clone 25 and 46) expressed medium and high level of maspin, respectively, while the control pS2-GFP clone had no detectable maspin expression. These clones were used for subsequent experiments. F1

Effects of maspin on cell proliferation in C2N tramp cells.

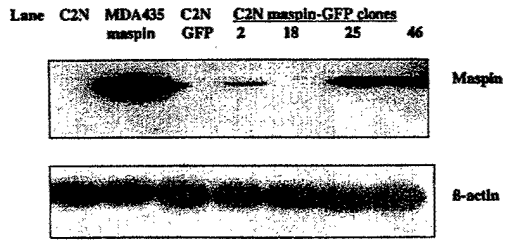

FIG. 1. Western blot analysis of C2N maspin-GFP stable transfectants. Cell lysates (50 μg.) from C2N stable transfectants were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, transferred onto polyvinylidine difluoride membrane and probed with mouse monoclonal antibody to human maspin. Band (42 kDa.) was identified in several clones. Blot was stripped and probed with β-actin antibodies for equal sample loading.

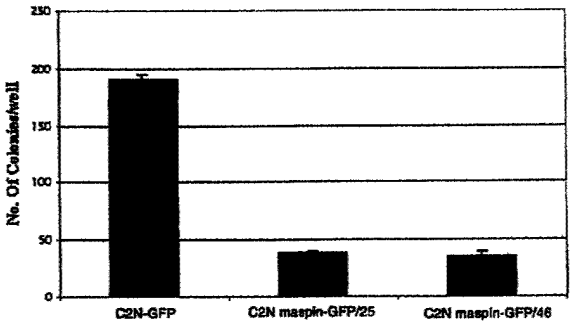

FIG. 3. Anchorage independent growth of maspin expressing C2N cells. Clones indicated were plated in soft agar in triplicate in 6-well plate and incubated at 37C in 5% CO_2 for 3 weeks. Cells were stained with p-nitrotetrazolium violet for 16 hours at 37C, counted and photographed using dissecting microscope linked to Nikon camera. Each sample was assayed in triplicate and experiment was repeated twice. Error bars represent ± SD.

To investigate the biological effects of maspin on invasive C2N cells we tested the growth rates of stable transfectants. When cells were grown in log phase for 2 to 5.2 days, a striking variation in the growth pattern was observed among stable transfectants on MTS assay. Cells reached confluence by days 6 to 7, after which the assay was stopped. Indeed, stable transfectants expressing maspin showed a lower growth rate than control transfectants (pS2-GFP) and the parental cell line. Moreover, the level of maspin expression determined growth inhibition in a dose dependent manner. Clone 2 expressing low levels of maspin had lower growth inhibition than high maspin expressing clones 25 and 46. The vector-only control clone had a growth pattern similar to that F2 of parental C2N cells (fig. 2). These data indicate that maspin was responsible for growth suppression.

Effect of maspin on anchorage independent growth of C2N cells. We determined the antimetastatic potential of maspin in vitro by soft agar assay. After plating 6×10^3 cells in triplicate in soft agar the number of colonies formed and colony size were analyzed at the end of 3 weeks. Colonies F3 greater than 1 mm. were scored. Figure 3 shows that GFP-vector only stable transfectants demonstrated a higher capability of colony formation in soft agar, while clones over expressing maspin showed a lower capability of colony formation ($p < 0.01$). Moreover, plating more cells or increasing incubation period did not increase the colonies of clones 25 and 46 (data not shown). These data indicate that maspin expression decreased the anchorage independent growth capacity of C2N cells.

Increased adhesion of maspin transfectants to ECM components fibronectin and laminin. Cell adhesion to ECM is a preliminary step involved in invasion and metastasis. ECM consists of a composite pool of matrix proteins with laminin and fibronectin as its major components. Therefore, we investigated the ability of stable transfectants to adhere to the ECM protein fibronectin or laminin. On adhesion assays maspin stable transfectants showed a 60% to 70% increase in binding to laminin and fibronectin compared with vector-only control transfectants (fig. 4). Such changes in adhesion to F4 laminin and fibronectin were statistically significant ($p < 0.02$ and < 0.01, respectively). These data demonstrate that maspin can indeed increase the ability of cells to adhere to ECM molecules, although in a dose independent manner.

Decreased chemo-invasion of maspin transfectants in vitro. The metastatic potential of tumors depends on the ability of the tumor cells to invade through the basement membrane and migrate to distant sites. We examined the ability of maspin transfectants to penetrate Matrigel using the modified Boyden chamber assay. Significant differences in cell invasion were observed in vector-only transfectants and

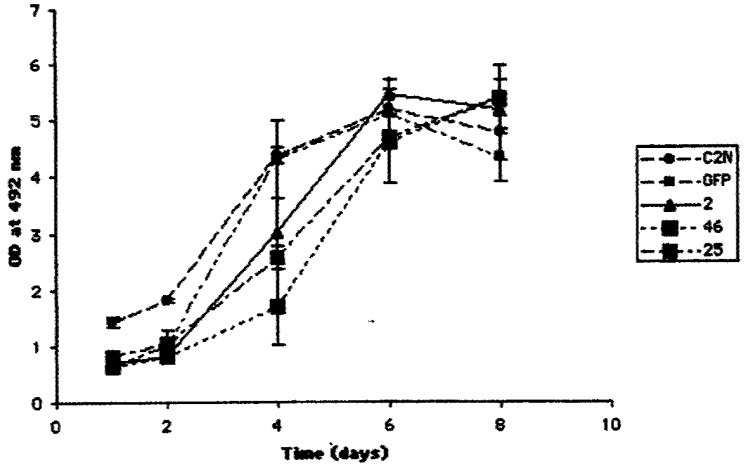

FIG. 2. Growth inhibition of C2N maspin-GFP expressing clones. Cells were seeded at 1×10^3 in 24-well plate and growth was measured by MTS assay on days indicated. Error bars represent ± SD of 3 experiments. *OD*, optical density.

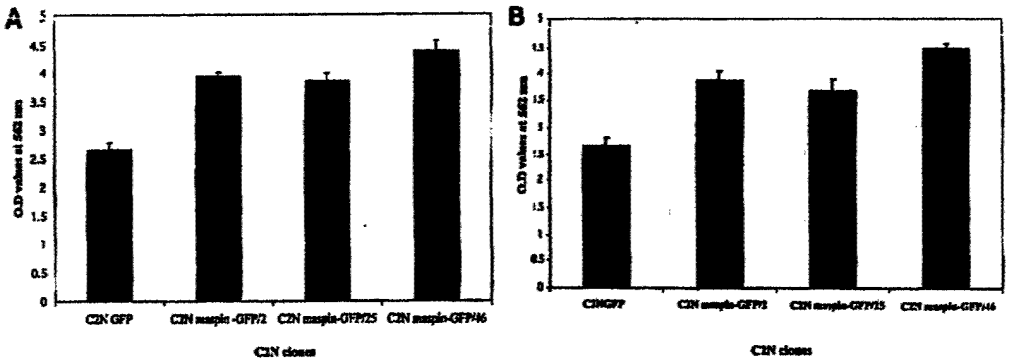

FIG. 4. Cell adhesion of stable transfectants to matrix proteins. *A*, adhesion assay of stable transfectants to fibronectin. Cells were seeded onto wells coated with 25 μg/ml. fibronectin and incubated at 37C for 2 hours. Cell density was measured by protein estimation using BCA reagent. *B*, adhesion assay of stable transfectants to laminin. Cells were seeded onto wells coated with 25 μg/ml. laminin and incubated at 37C for 2 hours. Cell density was measured by protein estimation using BCA reagent. Error bars represent ± SEM of 8 experiments done in triplicate ($p < 0.05$). *O.D.*, optical density.

maspin transfectants. The rate of invasion was significantly higher in vector-only transfectants than in stable transfectants expressing maspin (fig. 5). Clone 2 expressing a lower maspin level had a higher rate of invasion, similar to vector-only transfectants. A dose dependent decrease in invasion was observed ($p < 0.05$). These data show that maspin expressed in prostate tumor cells could actively inhibit the ability of their invasion through Matrigel.

DISCUSSION

In the current study the maspin gene was expressed endogenously in highly invasive C2N TRAMP cells by retrovirus infection. Clones 2, 25 and 46 were arbitrarily selected based on maspin expression (low, medium or high) at the protein level. Our data indicated that cells expressing maspin were less invasive in Matrigel and showed less ability to grow in 3 dimensions in soft agar assay than control cells. Maspin expression also seemed to increase cell-ECM contact as assessed. Together these findings suggest that the tumor suppressive effect of maspin in prostate tumors is most probably achieved through cell-ECM interactions.

Tumor invasion and metastasis represent a complex process involving protease degradation of the basement membrane, migration and invasion of tumor cells.[22] A key question is how maspin exerts its tumor suppressing function in cancer. Sheng et al presented evidence in which maspin interacted with fibrinogen associated tissue plasminogen activator and with cell surface associated urokinase plasminogen activator.[23] Seftor et al showed that maspin suppressed invasive phenotypes of human breast cancer cells by modulating their integrin expression.[7] Recently Blacque et al provided new evidence that maspin interacts with types I and III collagen directly, suggesting that maspin could exert its anti-invasion and antimetastatic function by modulating cell adhesion with the extracellular matrix.[24] Two major components of ECM in prostate and mammary glands are laminin and fibronectin. To test whether maspin expressing prostate tumor cells had different cell adhesion affinity to laminin and fibronectin than those without maspin or lower maspin expressing cells we performed a cell adhesion assay. In the presence of either matrix molecule maspin stable transfectants adhered more to matrix components than control cells. This result is consistent with a previous study in which MDA 231 breast tumor cells treated with maspin had increased integrin activity, which resulted in increased cell adhesion to fibronectin.[7] Moreover, Ngamkitidechakul et al noted that corneal stromal cells increased adhesion to fibronectin and laminin in the presence of exogenous maspin.[25] The increased adhesion of maspin expressing C2N cells would likely make them more attached to the ECM, thus, preventing tumor cells from migrating freely through the basement membrane or triggering ECM mediated signaling pathways

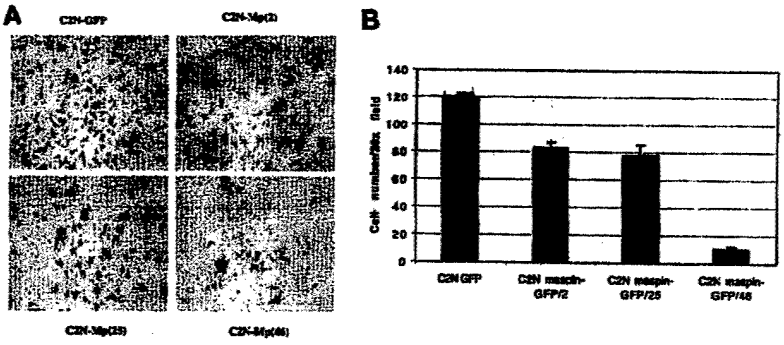

FIG. 5. Decreased cell invasion of C2N maspin stable transfectants. *A*, representative view of cells on chamber membrane. Cells were seeded onto filters coated Matrigel in modified Boyden chamber assay and incubated at 37C for 4 hours. Invaded cells were stained and counted under microscope. H & E, reduced from ×400. *B*, analysis of cell invasion assay. Error bars represent ± SD of 3 independent experiments (2-tailed t test $p < 0.05$).

that negatively regulate migration. In addition, using the Boyden chamber system we evaluated the effect of maspin on C2N cell invasion. Figure 5 shows that maspin expressing clones were greatly inhibited in their ability to invade through the matrix membrane. The difference was highly significant and reproducible. Thus, our data conclusively demonstrate that maspin can inhibit C2N tumor invasion in a fashion similar to that observed in breast tumors. Blocking tumor cell invasion and migration is an essential step for antimetastatic therapy because it limits tumor intravasation and extravasation.

CONCLUSIONS

Our study using TRAMP cells from the TRAMP mouse, an excellent model for prostate cancer, confirms the role of maspin as a tumor suppressor in prostate cancer. This study demonstrates decreased cell invasion in the presence of maspin, a phenomenon of paramount importance in metastasis. In fact, the tumor suppressive role of maspin is most likely achieved by preventing tumor cell invasion through the basement membrane and, thereby, preventing metastasis. Hence, the data further suggest that maspin can be a therapeutic target not only for breast cancer, but also for prostate cancer.

REFERENCES

1. Landis, S. H., Murray, T., Bolden, S. and Wingo, P. A.: Cancer statistics, 1998. CA Cancer J Clin, **48:** 6, 1998
2. Navone, N. M., Labate, M. E., Troncoso, P., Pisters, L. L., Conti, C. J., von Eschenbach, A. C. et al: p53 mutations in prostate cancer bone metastases suggest that selected p53 mutants in the primary site define foci with metastatic potential. J Urol, **161:** 304, 1999
3. Greenberg, N. M., DeMayo, F., Finegold, M. J., Medina, D., Tilley, W. D., Aspinall, J. O. et al: Prostate cancer in a transgenic mouse. Proc Natl Acad Sci USA, **92:** 3439, 1995
4. Zou, Z., Anisowicz, A., Hendrix, M. J., Thor, A., Neveu, M., Sheng, S. et al: Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science, **263:** 526, 1994
5. Sager, R., Sheng, S., Anisowicz, A., Sotiropoulou, G., Zou, Z., Stenman, G. et al: RNA genetics of breast cancer: maspin as paradigm. Cold Spring Harb Symp Quant Biol, **59:** 537, 1994
6. Zhang, M., Maass, N., Magit, D. and Sager, R.: Transactivation through Ets and Ap1 transcription sites determines the expression of the tumor-suppressing gene maspin. Cell Growth Differ, **8:** 179, 1997
7. Seftor, R. E., Seftor, E. A., Sheng, S., Pemberton, P. A., Sager, R. and Hendrix, M. J.: Maspin suppresses the invasive phenotype of human breast carcinoma. Cancer Res, **58:** 5681, 1998
8. Sheng, S., Carey, J., Seftor, E. A., Dias, L., Hendrix, M. J. and Sager, R.: Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. Proc Natl Acad Sci USA, **93:** 11669, 1996
9. Zhang, M., Volpert, O., Shi, Y. H. and Bouck, N.: Maspin is an angiogenesis inhibitor. Nat Med, **6:** 196, 2000
10. Xia, W., Lau, Y. K., Hu, M. C., Li, L., Johnston, D. A., Sheng, S. et al: High tumoral maspin expression is associated with improved survival of patients with oral squamous cell carcinoma. Oncogene, **19:** 2398, 2000
11. Jiang, W. G., Hiscox, S., Horrobin, D. F., Bryce, R. P. and Mansel, R. E.: Gamma linolenic acid regulates expression of maspin and the motility of cancer cells. Biochem Biophys Res Commun, **237:** 639, 1997
12. Mueller, E., Sarraf, P., Tontonoz, P., Evans, R. M., Martin, K. J., Zhang, M. et al: Terminal differentiation of human breast cancer through PPAR gamma. Mol Cell, **1:** 465, 1998
13. Zhang, M., Shi, Y., Magit, D., Furth, P. A. and Sager, R.: Reduced mammary tumor progression in WAP-TAg/WAP-maspin bitransgenic mice. Oncogene, **19:** 6053, 2000
14. Zhang, M., Magit, D., Botteri, F., Shi, Y., He, K., Li, M. et al: Maspin plays an important role in mammary gland development. Dev Biol, **215:** 278, 1999
15. Shi, H. Y., Zhang, W., Liang, R., Abraham, S., Kittrell, F. S., Medina, D. et al: Blocking tumor growth, invasion, and metastasis by maspin in a syngeneic breast cancer model. Cancer Res, **61:** 6945, 2001
16. Zhang, M., Magit, D. and Sager, R.: Expression of maspin in prostate cells is regulated by a positive ets element and a negative hormonal responsive element site recognized by androgen receptor. Proc Natl Acad Sci USA, **94:** 5673, 1997
17. Machtens, S., Serth, J., Bokemeyer, C., Bathke, W., Minssen, A., Kollmannsberger, C. et al: Expression of the p53 and Maspin protein in primary prostate cancer: correlation with clinical features. Int J Cancer, **95:** 337, 2001
18. Umekita, Y., Hiipakka, R. A. and Liao, S.: Rat and human maspins: structures, metastatic suppressor activity and mutation in prostate cancer cells. Cancer Lett, **113:** 87, 1997
19. Foster, B. A., Gingrich, J. R., Kwon, E. D., Madias, C. and Greenberg, N. M.: Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer Res, **57:** 3325, 1997
20. Armentano, D., Yu, S. F., Kantoff, P. W., von Ruden, T., Anderson, W. F. and Gilboa, E.: Effect of internal viral sequences on the utility of retroviral vectors. J Virol, **61:** 1647, 1987
21. Naviaux, R. K., Costanzi, E., Haas, M. and Verma, I. M.: The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. J Virol, **70:** 5701, 1996
22. Liotta, L. A., Steeg, P. S. and Stetler-Stevenson, W. G.: Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell, **64:** 327, 1991
23. Sheng, S., Truong, B., Fredrickson, D., Wu, R., Pardee, A. B. and Sager, R.: Tissue-type plasminogen activator is a target of the tumor suppressor gene maspin. Proc Natl Acad Sci USA, **95:** 499, 1998
24. Blacque, O. E. and Worrall, D. M.: Evidence for a direct interaction between the tumor suppressor serpin maspin, and types I and III collagen. J Biol Chem, **277:** 10783, 2002
25. Ngamkitidechakul, C., Burke, J. M., O'Brien, W. J. and Twining, S. S.: Maspin: synthesis by human cornea and regulation of in vitro stromal cell adhesion to extracellular matrix. Invest Ophthalmol Vis Sci, **42:** 3135, 2001